PRESENCE OF A CYCLIC-AMP-BINDING PROTEIN IN JERUSALEM ARTICHOKE RHIZOME TISSUES

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1. Introduction

Cyclic-AMP-binding protein has been found to occur in both animal [1] and bacterial cells (2–4) in which it has been shown to play different roles: in animal cells, it functions as a regulatory subunit of cAMP-dependent protein kinase systems [1], whereas in *Escherichia coli* it regulates gene transcription [5] by a mechanism that does not involve protein phosphorylation [6]. A cAMP-binding protein, whose function has not been determined, has also been reported in yeast [7] and rat liver [8].

Although both cAMP [9–13] and protein kinase activity [14–17] have been detected in higher plant tissues, neither cAMP-binding protein nor cAMP-dependent protein kinase have so far been reported. In this paper we report the presence of a cAMP-binding protein in Jerusalem artichoke rhizome tissues. However, this protein does not appear to be involved in protein kinase activity.

2. Methods

2.1. *Determination of protein kinase activity*

Protein kinase activity was determined, as previously reported [16], using [γ-^{32}P] ATP as a phosphate donor. Incubation was carried out at 30°C for 15 min.

2.2. *Determination of cAMP phosphodiesterase activity*

cAMP phosphodiesterase activity was determined as previously reported [18] except that the reaction mixture was the same as in the above protein kinase assay, but with 10 μM [^{3}H] cAMP instead of [γ-^{32}P] ATP.

2.3. *Determination of cAMP-binding activity*

cAMP-binding activity was determined according to Walton and Garren [19] with slight modifications. Each reaction mixture contained, in a total vol of 0.30 ml, 25 mM Tris–HCl (pH 7.6), 5 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 5×10^{-8} M [^{3}H] cAMP (0.43 μCi), 15 μg of protamine sulphate and tissue extracts. After incubation at 30°C for 15 min, the reaction was arrested by addition of 2 ml of cold 25 mM Tris–HCl (pH 7.6) containing 10 mM $MgCl_2$ and the mixture filtered through a Millipore filter which had been presoaked in the same buffer. The filter was washed 4 times with 5-ml portions of the same buffer, placed in a counting vial, dried and finally counted in a toluene–0.5% diphenyloxazole–0.005% 1,4-bis-[2-(5-phenyloxazole)] benzene scintillation liquid in a liquid scintillation spectrometer. Protamine was added to the reaction mixture according to Tao and Hackett [20] because it was found to improve (3–4 fold) the filter assay. Protamine itself did not bind appreciable amounts of cAMP. Moreover, we observed that protamine was effective even when added after incubation. This indicates that protamine improves retention of the cAMP-binding–protein complex on the filter rather than the cAMP-binding activity itself. The formation of a cAMP-binding–protein complex was also determined by gel chromatography on Sephadex G-25 (detail are reported in legend to fig.4).

2.4. *Preparation of cAMP-binding factor and protein kinase*

All operations were carried out 4°C. 50 g of peeled rhizomes were homogenized with 80 ml of 25 mM

Tris–HCl (pH 7.6) containing 5 mM $MgCl_2$ and 1 mM 2-mercaptoethanol. The homogenate was filtered through 3 layers of cheesecloth and the filtrate centrifuged at 105 000 *g* for 1 hr. The supernatant, containing 300 mg of protein in a total volume of 130 ml, was taken as the undialysed fraction. The solution was then dialysed overnight against 20 vol of extraction buffer and centrifuged at 10 000 *g* for 20 min to remove the precipitate which formed during dialysis. The clear supernatant (dialysed fraction), containing 150 mg of protein in a total vol of 150 ml, was brought to 50% saturation by slow addition of solid ammonium sulphate. The precipitate, containing both protein kinase and cAMP-binding activity, was collected by centrifugation and dissolved in 4 ml of extraction buffer. After desalting on a Sephadex G-25 column (1.5 X 30 cm) in the same buffer, the solution, containing 44 mg of protein, was chromatographed on a DEAE-cellulose column as described in the legend to fig.3.

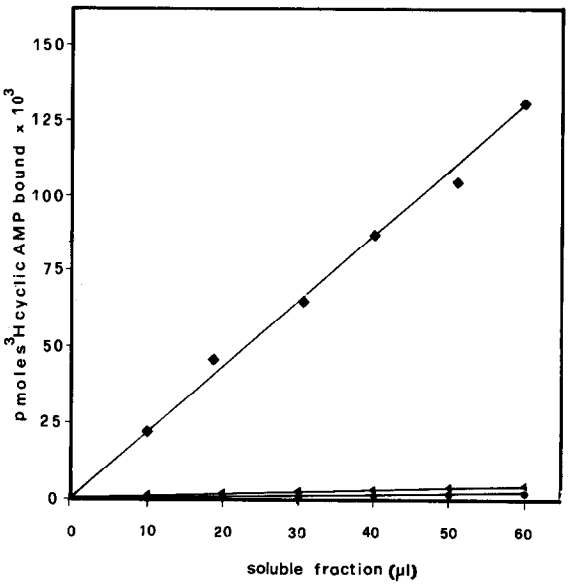

Fig.1. cAMP-binding activity of 105 000 *g* supernatant. Both undialysed and dialysed soluble fractions were prepared as described under Methods. Cyclic-AMP-binding activity was determined using Millipore filter technique. (●—●—●) undialysed fraction; (■—■—■) dialysed fraction; (▲—▲—▲) dialysed fraction with added an equal volume of undialysed one.

2.5. *Determination of protein concentration*

Protein concentration was determined according to Lowry et al. [21] except for that of the fractions eluting from the DEAE-cellulose column, which was determined by optical density at 280 and 260 nm [22].

3. Materials

Dormant rhizomes of Jerusalem artichoke (*Helianthus tuberosus* L. cv. Violet de Rennes) were harvested in November and stored at 4°C for 4–6 months before experimental use. All unlabelled nucleotides were obtained from Boehringer. Histone (type IIa) and phosvitin were purchased from Sigma; bovine albumin, protamine sulphate, casein from BDH; pronase and pancreatic DNAse from Calbiochem; pancreatic RNAse from Worthington; DEAE-cellulose (DE_{23}) from Whatman

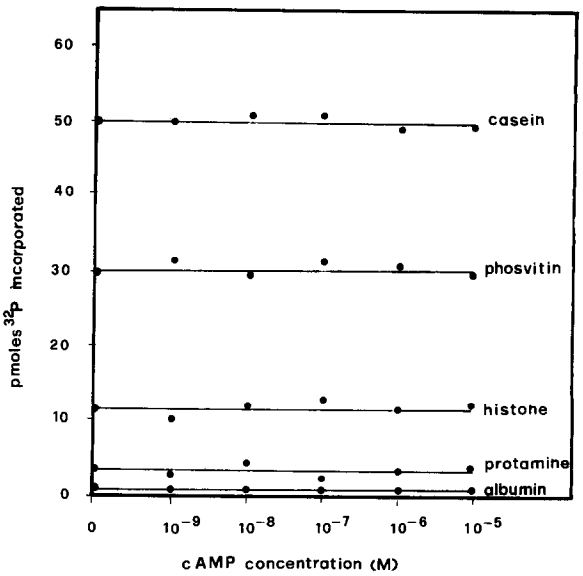

Fig.2. Effect of cAMP on protein kinase activity of dialysed 105 000 *g* supernatant. Protein kinase activity was assayed as reported under Methods. Each reaction mixture contained in a final vol of 0.2 ml: 40 mM Tris–HCl (pH 7.6); 10 mM $MgCl_2$; 5 mM 2-mercaptoethanol; 10 mM NaF; 20 µM [γ^{32}P] ATP (about 47 000 cpm/nmol); 200 µg of protein substrate and 40 µg of dialysed soluble fraction.

cellulose-ester membrane filters (HA 0.45 μ) from Millipore; diphenyloxazole, 1,4-bis-2-[(5-phenyloxazole)] benzene and Instagel from Packard. [γ-^{32}P] ATP (spec. activ. 17 Ci/mmole) and [8-^{3}H] cyclic-AMP (spec. activ. 27.5 Ci/mmole) were obtained from Amersham.

4. Results

As fig.1 shows, no cAMP activity could be detected in the undialysed soluble fraction. Activity appeared after dialysis and increased linearly with the quantity of fraction assayed. Moreover, when equal volumes of both undialysed and dialysed fractions were assayed together, activity again disappeared. The undialysed fraction retained its inhibitory property even when boiled for 3 min prior to addition (data not shown). The nature of this thermostable and dialysable inhibitor of cAMP-binding activity is currently under investigation.

In order to clarify whether the cAMP-binding activit was involved in the functioning of a cAMP-dependent protein kinase, we assayed the protein kinase activity of the dialysed fraction either in the presence or absence of cAMP. As shown in fig.2, although protein kinase activity towards different substrates could be detected, it was never affected by cAMP. The failure was apparently not due to cAMP degradation because

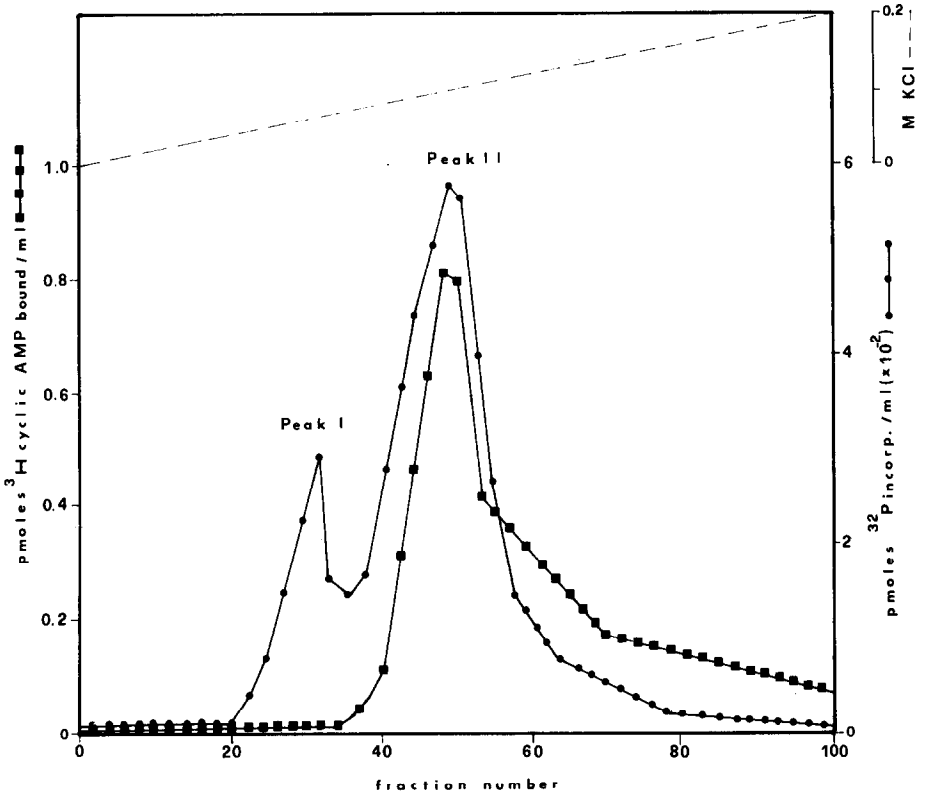

Fig.3. DEAE-cellulose column chromatography of cAMP-binding and protein kinase activity. The ammonium sulphate fraction, obtained as described under Methods and containing 44 mg of protein, was applied to a DEAE-cellulose column (2 × 6.5 cm) equilibrated with 25 mM Tris–HCl buffer (pH 7.6) containing 5 mM $MgCl_2$ and 1 mM 2-mercaptoethanol. The column was washed with 75 ml of the above buffer and then eluted with a linear 0–0.2 M KCl gradient in a total vol of 200 ml. Fractions of 2 ml were collected at a flow rate of 15 ml/hr. Cyclic-AMP-binding and protein kinase activity was determined as described under Methods. Casein was added to the reaction mixture for protein kinase assay at a concentration of 1 mg/ml. The peak fractions (24–32, peak I and 42–56, peak II) were pooled and stored at –20°C until used. The protein concentration of peaks I and II was 80 μg/ml and 20 μg/ml respectively (■–■–■) cAMP-binding activity; (●–●–●) protein kinase activity.

no phosphodiesterase activity could be detected when it was tested under the experimental conditions of the protein kinase assay. Similar results were obtained when protein kinase activity was assayed at pH 6.2 (data not shown).

To test the possibility that the presence of cAMP-dependent protein kinases was masked by the activity of cAMP-independent ones, the dialysed soluble extract was fractionated as described under Methods. Two peaks of protein kinase (assayed using casein as exogenous substrate) and one of cAMP-binding activity were resolved from the DEAE-cellulose column as shown in fig.3. Although the peak of cAMP-binding activity coincided with peak II of protein kinase, the latter was not affected by cAMP (in the range $10^{-9}-10^{-5}$ M). Protein kinase of peak II was also unaffected by cAMP, when, instead of casein, either phosvitin or histone was used as exogenous substrate. cAMP was also ineffective on the protein kinase activity of peak I.

We have used peak II to study some properties of the cAMP-binding factor. As table 1 indicates, the factor is a protein. Indeed, the activity was destroyed by pronase, but unaffected by DNAse or RNAse. Further evidence of its protein nature was obtained by Sephadex G-25 filtration of the [^{3}H]cAMP-binding–protein complex. As fig.4 shows, the radioactive com-

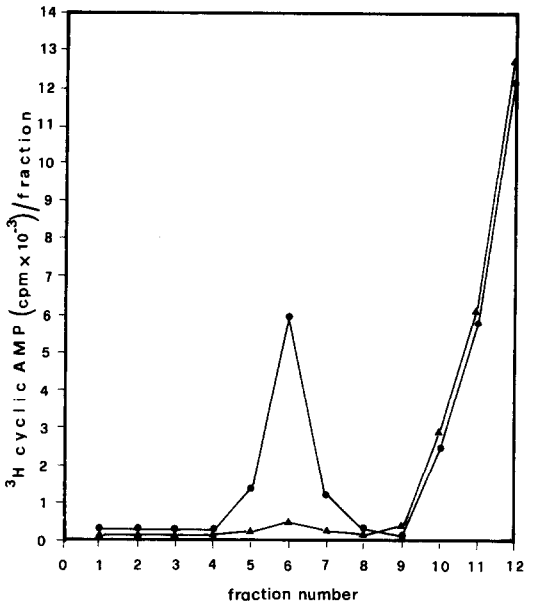

Fig.4. Sephadex G-25 filtration of the cAMP-binding factor complex. 0.6 ml-reaction mixture, containing 25 mM Tris–HCl buffer (pH 7.6), 5 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 10 μg of binding protein was incubated in the presence or absence of 25 μg of pronase for 30 min at 30°C. Afterwards, 0.86 μCi of [^{3}H]cAMP was added to the reaction mixture, that was further incubated for 15 min at 30°C. The mixture was then applied to a Sephadex G-25 column (0.9 × 13 cm), that has been equilibrated with 25 mM Tris–HCl buffer (pH 7.6) containing 5 mM $MgCl_2$ and 1 mM 2-mercaptoethanol, and eluted with the same buffer. 0.5 ml-fractions were collected and counted in Instagel in a liquid scintillation spectrometer. (●–●–●) untreated; (▲–▲–▲) pronase treated.

Table 1
Effect of various enzyme on the activity of the cAMP-binding factor.

Enzyme added	[^{3}H]cAMP bound (picomoles/4 μg protein)	Inhibition (%)
none	0.150	0
pronase (3.5 μg)	0.017	89
pronase (15 μg)	0.005	97
DNAse (15 μg)	0.150	0
RNAse (15 μg)	0.150	0

4 μg of protein from the DEAE-cellulose peak of cAMP-binding activity were incubated at 30°C for 30°C with or without the indicated enzymes in 0.30 ml-reaction mixture containing 25 mM Tris–HCl buffer (pH 7.6), 5 mM $MgCl_2$, and 1 mM 2-mercaptoethanol. After incubation 0.43 μCi of [^{3}H]cAMP (spec. act. 27.5 Ci/ mmole) was added to each reaction mixture and the mixture was further incubated for 15 min at 30°C. After incubation, 15 μg of protamine sulphate was added to each reaction mixture and [^{3}H]cAMP bound was determined using the Millipore membrane filtration technique.

Table 2
Effect of various nucleotides on binding of [^{3}H]cAMP.

Nucleotide added	[^{3}H]cAMP bound (picomoles/4 μg protein)	Inhibition (%)
none	0.160	0
3′,5′ cAMP	0.076	53
8 Br-3′,5′ cAMP	0.101	37
3′,5′ cGMP	0.160	0
3′,5′ cUMP	0.160	0
3′,5′ cIMP	0.160	0
3′,5′ cCMP	0.160	0
5′-AMP	0.160	0

Each reaction mixture contained 4 μg of protein from the DEAE-cellulose peak of cAMP-binding activity. Unlabeled nucleotides were added at the final concentration of 5×10^{-7} M. Binding activity was determined using the Millipore membrane filtration technique.

plex eluted with the exclusion peak and disappeared after pronase treatment of the binding factor.

In order to determine if cAMP was altered upon binding, the [^{3}H] cAMP-bound protein was separated on Sephadex G-25, added to 10^{-3} M unlabelled cAMP, boiled for 1 min and subjected to chromatography on PEI-cellulose thin-layer plates [18]. More than 90% of the radioactivity moved with cAMP.

Table 2 reports the affect of several nucleotides on [^{3}H]cAMP-binding activity. It is evident that unlabelled cAMP and its 8-Bromo derivative at a concentration of 5×10^{-7} M significantly inhibited the binding, whereas the other nucleotides tested were ineffective.

5. Discussion

We have previously reported the presence of cAMP [13] and of the enzymes of its metabolism [18,23] in dormant rhizomes of Jerusalem artichoke. The above data indicate that these tissues also contain a cAMP-binding protein. Attempts to show that this protein is involved in the functioning of cAMP-dependent protein kinase have failed. Indeed, although protein kinase and cAMP-binding activity were associated both in the crude soluble fraction and after DEAE-cellulose chromatography, protein kinase activity always remained unaffected by cyclic AMP.

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References

[1] Langan, T. A. (1973) in: Advances in Cyclic Nucleotide Research (Greengard, P. and Robinson, G. A., eds.), Vol. 3, pp. 99–153. Raven Press, New York.
[2] Zubay, G., Schwartz, D. and Becwith, J. (1970) Proc. Natl. Acad. Sci. 66, 104–110.
[3] Emmer, M., DeCrombrugghe, B., Pastan, I. and Perlman, R. L. (1970) Proc. Natl. Acad. Sci. 66, 480–487.
[4] Anderson, W. B., Scneider, A. B., Emmer, M., Perlman, R. L. and Pastan, I. (1971) J. Biol. Chem. 246, 5929–5937.
[5] DeCombrugghe, B., Chen, B., Anderson, W. B., Gottesmai M. E., Perlman, R. L. and Pastan, I. (1971) J. Biol. Chem. 246, 7343–7348.
[6] Pastan, I. and Perlman, R. L. (1972) in: Advances in Cyclic Nucleotide Research (Greengard, P. and Robinson, G. A., eds.), Vol. 1, pp. 11–16, Raven Press, New York.
[7] Sy, J. and Richter, D. (1972) Biochemistry 11, 2784–2787.
[8] Chambault, A. M., Leray, F. and Hanoune, J. (1971) FEBS Lett. 15, 328–334.
[9] Raymond, P., Narayanan, A. and Pradet, A. (1973) Biochem. Biophys. Res. Commun. 53, 1115–1121.
[10] Brewin, N. J. and Northcote, D. H. (1973) J. Exper. Bot. 24, 881–888.
[11] Brown, E. G. and Netwon, R. P. (1973) Phytochemistry 12, 2683–2685.
[12] Lunden, C. V., Wood, H. N. and Braun, A. C. (1973) Differentiation 1, 255–260.
[13] Giannattasio, M., Mandato, E. and Macchia, V. (1974) Biochem. Biophys. Res. Commun. 57, 365–371.
[14] Ralph, R. K., McCombs, P. J. A., Tener, G. and Wojicik, S. J. (1972) Biochem. J. 130, 901–911.
[15] Keates, R. A. B. (1973) Biochem. Biophys. Res. Commun 54, 655–661.
[16] Carratù, G., Manzocchi, L. A., Lanzani, G. A. and Giannattasio, M. (1974) Plant Science Lett. 3 (in press).
[17] Giannattasio, M., Carratù, G. and Tucci, G. F. (1974) Proc. Meeting of Italian Soc. of Plant physiology, Florenc
[18] Giannattasio, M., Sica, G. and Macchia, V. (1974) Phytochemistry 13 (in press).
[19] Walton, G. M. and Garren, L. D. (1970) Biochemistry 9, 4223–4229.
[20] Tao, M. and Hackett, P. (1973) J. Biol. Chem. 248, 5324–5332.
[21] Lowry, O. H., Rosenbrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–274.
[22] Colowick, S. P. and Kaplan, N. D. (1957) Methods in Enzymology, 3, 254–457.
[23] Giannattasio, M. and Macchia, V. (1973) Plant Science Lett. 1, 259–264.